AVIAN DISEASES 53:396–404, 2009

Induction of CXC Chemokine Messenger-RNA Expression in Chicken Oviduct Epithelial Cells by *Salmonella enterica* Serovar Enteritidis via the Type Three Secretion System–1

Shuhui Li,[A] M. Zhenyu Zhang,[A] Lifang Yan,[A] Hyun Lillehoj,[B] Lanny W. Pace,[A] and Shuping Zhang[AC]

[A]Mississippi Veterinary Research and Diagnostic Laboratory, Department of Pathobiology and Population Medicine, College of Veterinary Medicine, Mississippi State University, P.O. Box 97813, Pearl, MS 39288
[B]Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, U.S. Department of Agriculture, Beltsville, MD 20705

Received 11 February 2009; Accepted and published ahead of print 19 April 2009

SUMMARY. The messenger-RNA (mRNA) expression of selected cytokines and chemokines in primary chicken oviduct epithelial cells (COEC) was determined following *in vitro* infections with wild-type or type three secretion system (T3SS)–mutant *Salmonella enterica* serovar Enteritidis (SE) strains. All SE strains examined in this study elicited the expression of proinflammatory immune mediators including inducible nitric oxide synthase (iNOS), CXCLi1 (K60), CXCLi2 (IL-8), CCLi3 (K203), and CCLi4 (MIP-1β). SE also triggered the expression of an anti-inflammatory cytokine, IL-10, but repressed TGF-β3 transcription. Both T3SS-1 (*sipA* and *sipB*) and T3SS-2 (*pipB* and *ssaV*) mutants showed reduced capacity, compared to the wild-type SE, to stimulate iNOS mRNA expression in COEC. T3SS-1 (*sipA* and *sipB*) mutants were significantly impaired in their ability to induce the expression of CXCLi1 and CXCLi2. T3SS-2 mutants displayed a wild-type phenotype in terms of modulating the expression of chemokines and cytokines in COEC. The expression of iNOS, but not CXC chemokines, correlated with the number of intracellular bacteria in COEC. Genetic complementation of the *sipA* mutation restored a wild-type phenotype. Thus, SE induction of CXCLi1 and CXCLi2 was *sipA*-dependent. These results provide enhanced insights into the complex interplay between local host innate immune system and bacterial virulence factors.

RESUMEN. Inducción de la expresión del ARN mensajero de la quimioquina CXC en células epiteliales de oviducto de pollo por *Salmonellla enterica* Serovar Enteritidis a través del sistema de secreción tipo III-1.

La expresión de ARN mensajero de citoquinas y qumioquinas selectas fue determinada en cultivos primarios de células epiteliales de oviducto después de la infección *in vitro* con cepas silvestres y cepas mutantes del sistema de secreción tipo III (con las siglas en inglés T3SS) de *Salmonella enterica* serovar Enteritidis (SE). Todas las cepas de SE examinadas en este estudio estimularon la producción de mediadores inmunes proinflamatorios incluyendo a la enzima óxido nitrico sintetasa inducible (con las siglas en inglés iNOS), CXCLi1 (K60), CXCLi2 (IL-8), CCLi3 (K203) y CCLi4 (MIP-1β). También, la SE estimuló la expresión de la citoquina antiinflamatoria IL-10, pero reprimió la transcripción de TGF-β3. Ambas bacterias mutantes, T3SS-1 (con genes *sipA* y *sipB*) y T3SS-2 (con genes *pipB* y *ssaV*) mostraron una capacidad reducida en comparación con la cepa silvestre de SE para estimular la expresión de ARN mensajero de la enzima iNOS en las células epiteliales de oviducto de pollo. Las bacterias mutantes T3SS-1 (*sipA* y *sipB*) mostraron una capacidad disminuida para inducir la expresión de CXCLi1 y CXCLi2. Los mutantes T3SS-2 mostraron un fenotipo similar al silvestre en términos de la modulación de la expresión de quimioquinas y citoquinas en las células epiteliales de oviducto de pollo. Sólo la expresión de la iNOS y no de otras quimioquinas CXC, estuvo correlacionada con el número de bacterias intracelulares en las células epiteliales de oviducto de pollo. La complementación genética de la mutación *sipA* re-estableció el fenotipo silvestre. De esta manera, la inducción por SE de CXCLi1 y CXCLi2 fue dependiente del gen *sipA*. Estos resultados proporcionan una mayor información acerca de las relaciones complejas entre el sistema inmune innato del hospedador y los factores de virulencia bacterianos.

Key words: *Salmonella*, chemokines, cytokines, oviduct epithelial cells

Abbreviations: cDNA = complementary DNA; CFU = colony-forming units; COEC = chicken oviduct epithelial cells; Ct = cycle threshold; ELISA = enzyme-linked immunosorbent assay; HBSS = Hanks balanced salt solution; hpi = hours post infection; IL = interleukin; INF = interferon; iNOS = inducible nitric oxide synthase; LB broth = Luria-Bertani broth; LDH = lactate dehydrogenase; mRNA = messenger RNA; MEM = minimal essential medium; MIP = macrophage inflammatory protein; MOI = multiplicity of infection; PAMP = pathogen-associated molecular patterns; PMN = polymorphonuclear leukocytes; RT-PCR = reverse transcriptase-PCR; SE = *Salmonella* Enteritidis; SPI = *Salmonella* pathogenicity island; T3SS = type three secretion system; TGF = transforming growth factor

Salmonella enterica serovar Enteritidis (SE) has a broad range of hosts including mammals and poultry (1,23). SE causes systemic infection in newly hatched chicks characterized by anorexia, depression, drowsiness, and infrequent mortality (1). In older chickens, SE infection often results in intestinal and/or reproductive tract colonization without causing overt clinical signs (2,14). Intestinal-carrier state is a significant source of horizontal transmission of *Salmonella* whereas reproductive tract colonization contributes to SE contamination of eggs and hatcheries (7,11,12,20). Within the upper oviduct, the isthmus is the predominant colonization site of SE (8). To understand the protective immunity against *Salmonella*, many investigations have focused on the expression of immune response genes (22,25,30,31,36). It has been demonstrated that the expression of proinflammatory and T-helper cell type 1 cytokines and subsequent recruitment of T cells to the site of infection are prerequisites to a cell-mediated clearance of *Salmonella* (21,25). To date, the expression of interferon (INF)-γ, interleukin (IL)-2, IL-6, CXCLi1 (K60), CXCLi2 (IL-8), or CXCL4

[C]Corresponding author. E-mail: zhang@cvm.msstate.edu

Table 1. SE strains used in this study.

Strain	Relevant genotype	Reference
ZM100	Derivative of SE338 *nal*^{r}	18
ZM101	ZM100 *nal*^{r} *cm*^{r} *sipB::pEP185.2*	18
ZM102	ZM100 *nal*^{r} *cm*^{r} *ssaV::pEP185.2*	18
ZM103	ZM100 *nal*^{r} *cm*^{r} *sipA::pEP185.2*	18
ZM106	ZM100 *nal*^{r} *cm*^{r} *pipB::pEP185.2*	18
ZM103C	Derivative of ZM103, *pSipA*	18

(MIP-1β), etc. has been examined in chicken systemic organs (spleen and liver), intestinal tissue (cecum), immune cells (macrophages and heterophils), and primary cultures of chick kidney cells (6,25,30,32). Although a significant amount of data regarding systemic and intestinal immune response to *Salmonella* has been generated, the reproductive tract immunity against SE colonization is less known.

Asymptomatic colonization involves a dynamic balance between the host response and bacterial virulence. The members of *Salmonella enterica* possess two virulence-associated type three secretion systems (T3SS-1 and T3SS-2) encoded by *Salmonella* pathogenicity island (SPI)-1 and SPI-2, respectively (10,29). The T3SSs consist of regulatory proteins, structural proteins forming a needle-like apparatus, translocases necessary for the delivery of effectors, and secreted effectors that modulate host cellular events (13,24). T3SS-1 effectors act in concert to facilitate bacterial entry into epithelial cells whereas T3SS-2 effectors promote bacterial survival or replication within host phagocytes (5,27). In addition, T3SS-1 effectors differentially modulate cytokine response, which shapes the type and degree of pathologic changes associated with *Salmonella* infections in different mammalian host species (26,33). Recent studies have demonstrated that T3SS-1 and T3SS-2 are required by *Salmonella enterica* to colonize chicken intestinal and/or reproductive tissues (3,4,15). The work from our laboratory shows that both T3SS-1 and T3SS-2 are necessary for the entry of SE into chicken oviduct epithelial cells (COEC) (18). To understand the immunologic mechanisms underlying SE colonization of the chicken reproductive tract, we determined the messenger RNA (mRNA) expression profiles of selected cytokines and chemokines in primary COEC infected with either the wild-type SE or individual T3SS-1 and T3SS-2 mutant SE strains. The goal of this study was to characterize the roles of T3SS-1 and T3SS-2 in modulating host cytokine and chemokine responses in SE-infected oviduct epithelial cells.

MATERIALS AND METHODS

SE. SE strains used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) broth containing 50 μg/ml nalidixic acid at 37 C for 16 to 24 hr. To prepare inocula for COEC, 50 μl of an overnight culture of each SE strain was diluted into 5 ml of fresh LB broth and incubated aerobically at 37 C for 4 hr. SE cultures at the logarithmic phase of growth were harvested by centrifugation for 10 min at 1500 × *g* and resuspended in fresh Hanks balanced salt solution (HBSS) without antibiotics at a concentration of 4×10^6/ml. The number of bacteria in each culture was determined by measuring the density at OD600 and confirmed by subsequent colony-forming unit (CFU) enumerations.

Cell cultures and infections. Primary COEC were prepared as described previously (18). In brief, fragments of isthmal epithelium of 25-wk-old broiler breeder hens (Ross-308) were first treated with collagenase (1 mg/ml) at 37 C for 30 min and then digested with 0.25% trypsin containing 3 mM ethylenediaminetetraacetic acid (Invitrogen, Carlsbad, CA) at 37 C for 10 min. Epithelial cells were collected by filtering the supernatant through cell strainers (100 μM) to remove tissue clumps. The epithelial cells and cell sheets quickly formed aggregates and stromal cells remained as single cells in the suspension. To separate epithelial cells from stromal cells, the cell suspension was centrifuged at 50 × *g* for 5 min. The loose pellet containing epithelial cells and small cell sheets was resuspended in minimal essential medium (MEM; ATCC, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum, 2% heat-inactivated chicken serum, insulin (0.12 U/ml, Sigma-Aldrich, St. Louis, MO), and estradiol benzoate (50 nM, Sigma-Aldrich). The cell suspension was then incubated in petri dishes at 40 C for 2 hr to allow the attachment of fibroblasts. The epithelial cells were collected by gentle pipetting and the number of cells was determined by trypan blue dye exclusion. Cells were seeded into 96-well tissue culture plates at a density of 2×10^4 cells/well and incubated at 40 C in 5% CO_2 for 48 hr. The epithelial origin of the COEC cultures was confirmed by immunocytochemistry to locate cytokeratin filaments (an epithelial cell marker) as described previously (18). Cultures with more than 80% cytokeratin-positive cells were used in subsequent infections.

Prior to infection, cell cultures were washed three times with fresh HBSS without antibiotics. For each bacterial strain and each time point, 100 μl of bacterial suspension containing 4×10^5 CFU was added into each of the eight replicate wells (2×10^4 COEC/well) to reach a multiplicity of infection (MOI) of 20:1 (bacteria:cell). The inoculated cultures were incubated at 40 C in 5% CO_2 for 1 hr, washed three times with fresh HBSS, and treated with 100 μg/ml gentamicin for 1 hr at 40 C to remove extracellular bacteria. After gentamicin treatment, triplicate wells were then incubated with 100 μl of 0.2% Triton X-100 (Sigma-Aldrich) for bacterial counting, another three wells were incubated with 100 μl of Trizol for RNA extraction, and the remaining two wells were used for assessment of COEC apoptosis. This time point was designated as 1 hr postinfection (hpi; T1). The remaining cell cultures were maintained in supplemented MEM (for COEC) containing 50 μg/ml gentamicin for an additional 3 hr and 23 hr followed by lysis for bacterial counting, enzyme-linked immunosorbent assay (ELISA), or RNA extraction. These time points were designated as 4 hpi (T4) and 24 hpi (T24), respectively. To determine the number of intracellular bacteria at each time point, 10-fold serial dilutions of the cell lysates (Triton X-100) were plated onto LB agar plates and incubated overnight at 37 C. The number of intracellular bacteria was converted into $\log_{10}$ CFU/ml. Infection experiments were repeated three times.

Cell death detection ELISA. SE-induced apoptosis of COEC was assessed using the Cell Death Detection ELISA Plus system (Roche Applied Science, Indianapolis, IN), a spectrophotometric enzyme immunoassay for the *in vitro* determination of cytoplasmic histone-associated DNA fragments of mono- and oligonucleosomes. Briefly, COEC were treated with the lysis buffer for 30 min at room temperature and centrifuged at 200 × *g* for 10 min. Cell lysate was transferred to the streptavidin-coated microplate and incubated with anti-histone and anti-DNA antibodies for 2 hr at room temperature. The antibody-nucleosome complexes bound to the microplates were incubated with peroxidase substrate for 15 min at room temperature. The absorbance at 405 nm was then determined. SE-induced apoptosis, expressed as an enrichment factor of mono- and oligonucleosomes in the cytoplasm of COEC, was calculated according to the following formula: (absorbance of the infected COEC) − (absorbance of the background)/(absorbance of control COEC) − (absorbance of the background). Experiments were repeated three times with replicate wells for each treatment group at each time point. Data generated from three independent experiments were presented as mean ± SD.

Lactate dehydrogenase (LDH) assay for cytotoxicity. LDH activity in the culture supernatant, an indicator of cell necrosis, was measured using the colorimetric Cytotoxicity Detection Kit (Roche Applied Science). In brief, 100 μl of freshly prepared reaction mixture was mixed with 100 μl of culture supernatant in a 96-well plate and incubated for 20 min at room temperature. The absorbance of the samples at 490 nm was measured. The percentage of cytotoxicity was calculated according to the formula: 100 × [(experimental release − spontaneous release)/(total release − spontaneous release)], in which spontaneous release is

Table 2. Primers used to amplify chicken cytokine and chemokine genes.

Primer	Sequence	Amplicon size
β-actin-F	5′-TGCGTGACATCAAGGAGAAG-3′	111 bp
β-actin-R	5′-GACCATCAGGGAGTTCATAGC-3′	
iNOS-F	5′-AGTTTGAAATCCAGTCGTGTTAC-3′	87 bp
iNOS-R	5′-AATATGTTCTCCAGGCAGGTAG-3′	
CXCli1-F	5′-GATGATGGGCAAGGCTGTAG-3′	106 bp
CXCLi1-R	5′-CTGGCATCGGAGTTCAATCG-3′	
CXCLi8-F	5′-GTAGGACGCTGGTAAAGATGG-3′	75 bp
CXCLi8-R	5′-TAGGGTGGATGAACTTAGAATGAG-3′	
CCLi3-F	5′-TCTTCTCATCGCATCCTTCTG-3′	93 bp
CCLi3-R	5′-ATCTTGTGTGTTATGTAAGTGGTG-3′	
CCLi4-F	5′-CAGACTACTACGAGACCAACAG-3′	137 bp
CCLi4-R	5′-GCATCAGTTCAGTTCCATCTTG-3′	
IL-10-F	5′-AGCCATCAAGCAGATCAAGG-3′	125 bp
IL-10-R	5′-ACTTCCTCCTCCTCATCAGC-3′	
TGF-β3-F	5′-ACGACACAAAGACCACACTC-3′	84 bp
TGF-β3-R	5′-CATTCAGATACACAGCAGTTCC-3′	

Table 3. Intracellular bacterial load in SE-infected COEC.[A]

	Log_{10} CFU ± SD per ml		
Strain (genotype)	1 hpi	4 hpi	24 hpi
ZM100 (wild type)	5.69 ± 0.04	5.75 ± 0.06	5.51 ± 0.10
ZM101 (*sipB*)	4.88 ± 0.16*	4.74 ± 0.31*	4.55 ± 0.36*
ZM103 (*sipA*)	5.37 ± 0.06*	5.26 ± 0.13*	4.84 ± 0.27*
ZM102 (*ssaV*)	5.29 ± 0.08*	5.51 ± 0.22	5.63 ± 0.05
ZM106 (*pipB*)	5.36 ± 0.02*	5.17 ± 0.55	5.36 ± 0.03

[A]COEC was infected with individual SE strains at MOI of 20:1 (bacteria:cell) as described in Materials and Methods section. Intracellular bacterial load presented as log CFU/ml was determined based on the results of three independent experiments with triplicate wells in each experiment.

*Indicates a significant reduction in the number of intracellular mutant bacteria compared to that of the wild-type strain, ZM100.

the amount of LDH activity in the supernatant of uninfected cells, and total release is the LDH activity in COEC lysates. Experiments were repeated three times with replicate wells for each treatment group at each time point. Data generated from three independent experiments were presented as mean ± SD.

RNA extraction and reverse transcriptase (RT)-PCR. Following Trizol treatment, the cell lysates in triplicate wells were combined. Total RNA was extracted from 1.2×10^6 COEC using the Trizol method (Invitrogen). RT-PCR was conducted using MultiScribe reverse transcriptase (Invitrogen) and the SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturers' instructions. The primer sequences of chicken β-actin, chemokine, and cytokine genes were obtained from the Entrez Nucleotide database and are listed in Table 2. Reverse transcription of total RNA (2 μg) in a volume of 100 μl containing 5.5 mM $MgCl_2$, 500 μM dNTP, 2.5 μM random hexamers, and 1.25 U of MultiScribe reverse transcriptase was performed at 42 C for 30 min. The resultant complementary DNA (cDNA) product was used as a template (4 μl/reaction) for subsequent real-time PCR in an ABI Prism 7700 thermocycler. PCR was carried out in a volume of 25 μl under the following conditions: 95 C for 10 min followed by 45 amplification cycles of 95 C for 15 sec, 58 C for 30 sec, and 72 C for 30 sec in the presence of 1× SYBR® Green PCR Master Mix. To determine SE-induced alterations in mRNA expression levels of immune mediators, the amount of β-actin cDNA was used to normalize the cDNA concentrations of different samples. The suitability of β-actin as a housekeeping gene internal control was determined by comparing the cycle threshold (Ct) values of RT-PCR using RNA prepared from SE-infected COEC at different time points as well as uninfected COEC cultures. The normalized amount of transcripts in SE-infected COEC relative to the amount of transcripts present in control COEC at each time point was calculated as fold-change using the formula $2^{-\Delta\Delta Ct \pm SD}$ (User bulletin No. 2, Applied Biosystems).

Statistical analysis. The difference in number of intracellular bacteria, COEC apoptosis and necrosis, or mRNA expression of immune genes between the wild-type SE and each of the T3SS mutants at each time point was analyzed by performing an analysis of variance with PROC GLM (SAS 9.1) and DUNNET mean comparison tests. The strength of association between the number of intracellular bacteria

Table 4. Apoptosis of COEC following infections with different SE strains.

	Enrichment factor of COEC[A]		
Strain (genotype)	1 hpi	4 hpi	24 hpi
ZM100 (wild type)	1.95 ± 1.27	1.72 ± 0.45	4.89 ± 2.15[δ]
ZM101 (*sipB*)	1.48 ± 0.63	1.12 ± 0.25	4.25 ± 1.62[δ]
ZM103 (*sipA*)	1.31 ± 0.29	1.54 ± 0.40	4.61 ± 2.19[δ]
ZM102 (*ssaV*)	1.73 ± 0.58	1.45 ± 0.52	5.07 ± 2.11[δ]
ZM106 (*pipB*)	1.63 ± 0.66	1.55 ± 0.59	4.43 ± 2.43[δ]

[A]Enrichment factor was a parameter of apoptosis indicated by the release of mono- and oligonucleosomes into COEC cytoplasm.

[δ]Indicates a significant increase in the apoptotic death of COEC between 1 hpi and 24 hpi.

Table 5. Necrosis of COEC following infections with different SE strains.

Strain (genotype)	Percentage of LDH release[A]		
	1 hpi	4 hpi	24 hpi
ZM100 (wild type)	1.98 ± 0.1	4.86 ± 3.54	9.02 ± 1.36[δ]
ZM101 (*sipB*)	2.04 ± 0.10	4.65 ± 1.44	8.21 ± 1.21[δ]
ZM103 (*sipA*)	2.66 ± 1.05	4.45 ± 2.83	8.98 ± 1.68[δ]
ZM102 (*ssaV*)	1.83 ± 1.00	3.92 ± 1.88	8.18 ± 0.72[δ]
ZM106 (*pipB*)	1.98 ± 0.66	6.32 ± 3.27	9.49 ± 0.72[δ]

[A]Percentage of LDH release by COEC exposed to SE was calculated based on the ratio of LDH in the supernatant and that in the COEC lysates.
[δ]Indicates a significant increase in the necrotic death of COEC between 1 hpi and 24 hpi.

Table 6. β-actin mRNA expression in SE-infected and control COEC.

Infecting strain (genotype)	RT-PCR Ct values[A]		
	1 hpi	4 hpi	24 hpi
ZM100 (wild type)	25.79 ± 1.45	25.89 ± 0.98	24.58 ± 0.95
ZM101 (*sipB*)	25.06 ± 0.90	225.82 ± 1.29	24.24 ± 0.38
ZM103 (*sipA*)	25.61 ± 1.06	25.64 ± 1.16	24.22 ± 0.26
ZM102 (*ssaV*)	25.52 ± 1.69	25.69 ± 1.18	24.79 ± 0.45
ZM106 (*pipB*)	25.46 ± 1.07	25.68 ± 1.45	24.91 ± 0.63
Uninfected control	25.87 ± 0.44	25.80 ± 1.57	25.80 ± 1.12

[A]Total RNA extraction and RT-PCR were conducted as described in Materials and Methods section. Data shown are Ct values (means ± SD) from three independent infections. No significant differences in the expression of β-actin were detected among treatment groups.

and the mRNA expression level at a given time point was determined using linear regression (PROC REG, SAS 9.1).

RESULTS

Intracellular bacterial load and COEC death. At 1 hpi, the number of each T3SS mutant recovered from COEC was significantly lower than that of the wild-type SE, indicating an impaired invasiveness of the mutants (Table 3). At 4 hpi and 24 hpi, the numbers of T3SS-1 mutants (*sipB* or *sipA*), but not T3SS-2 mutants (*ssaV* or *pipB*), were still significantly lower than that of the wild-type strain. No significant difference in apoptosis was detected between COEC infected with the wild-type SE and those infected with individual T3SS mutants at any particular time points (Table 4). Similarly, no significant differences in necrosis were detected among COEC cultures infected with different SE strains (Table 5). A significant increase in COEC death was observed in all treatment groups at 24 hpi, compared to that at 1 hpi or 4 hpi (Tables 4, 5).

The mRNA expression of cytokines and chemokines in SE-infected COEC. Validity of β-actin as the internal control gene was confirmed by the stability of its expression levels (similar Ct values) across the sample panel (Table 6). SE strains examined in this study elicited the expression of proinflammatory immune mediators in

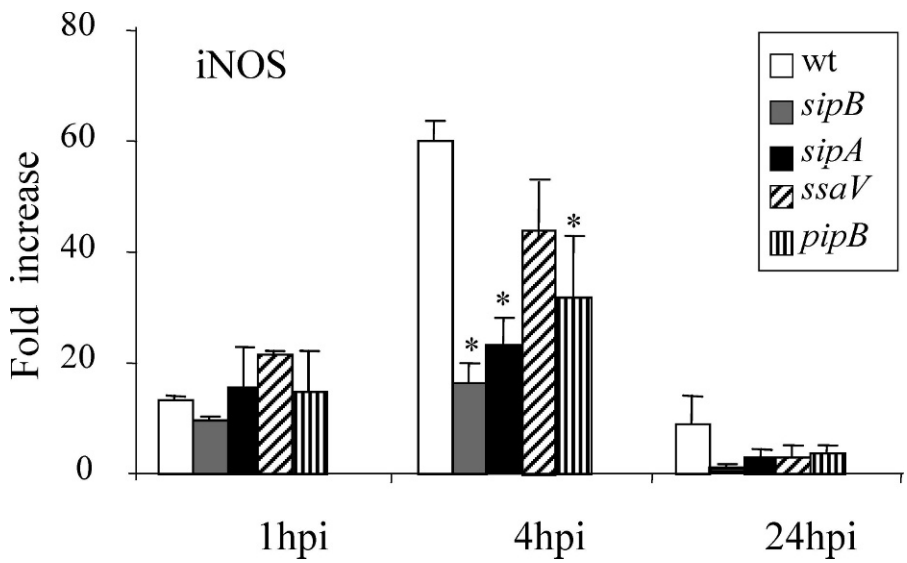

Fig. 1. SE-induced iNOS mRNA expression in COEC. COEC were infected with wild-type or *sipB*-, *sipA*-, *ssaV*-, or *pipB*-mutant SE at an MOI of 20:1. The amount of iNOS transcripts in SE-infected COEC relative to that in the control was determined by RT-PCR and presented as fold increase. Duplicate reactions were included in RT-PCR. Data shown (means ± SD) are from three independent experiments. * indicates the difference between the wild type and the mutant was significant ($P < 0.05$).

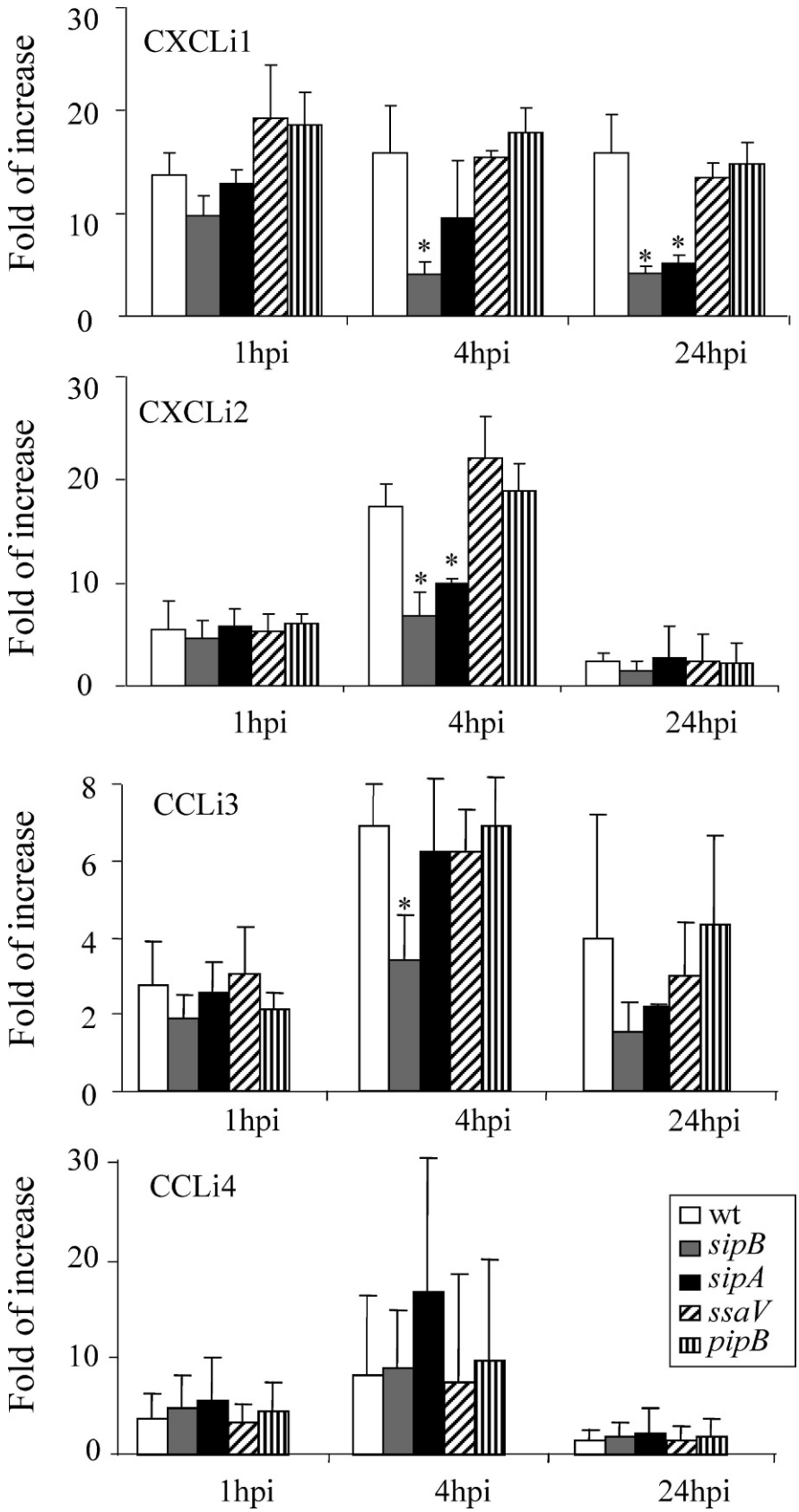

Fig. 2. SE-induced the expression of proinflammatory chemokines in COEC. The amount of chemokine transcripts in SE-infected COEC relative to that in the control was determined by RT-PCR and presented as fold increase. Duplicate reactions were included in RT-PCR. Data shown (means ± SD) are from three independent experiments. * indicates the difference between the wild type and the mutant was significant ($P < 0.05$).

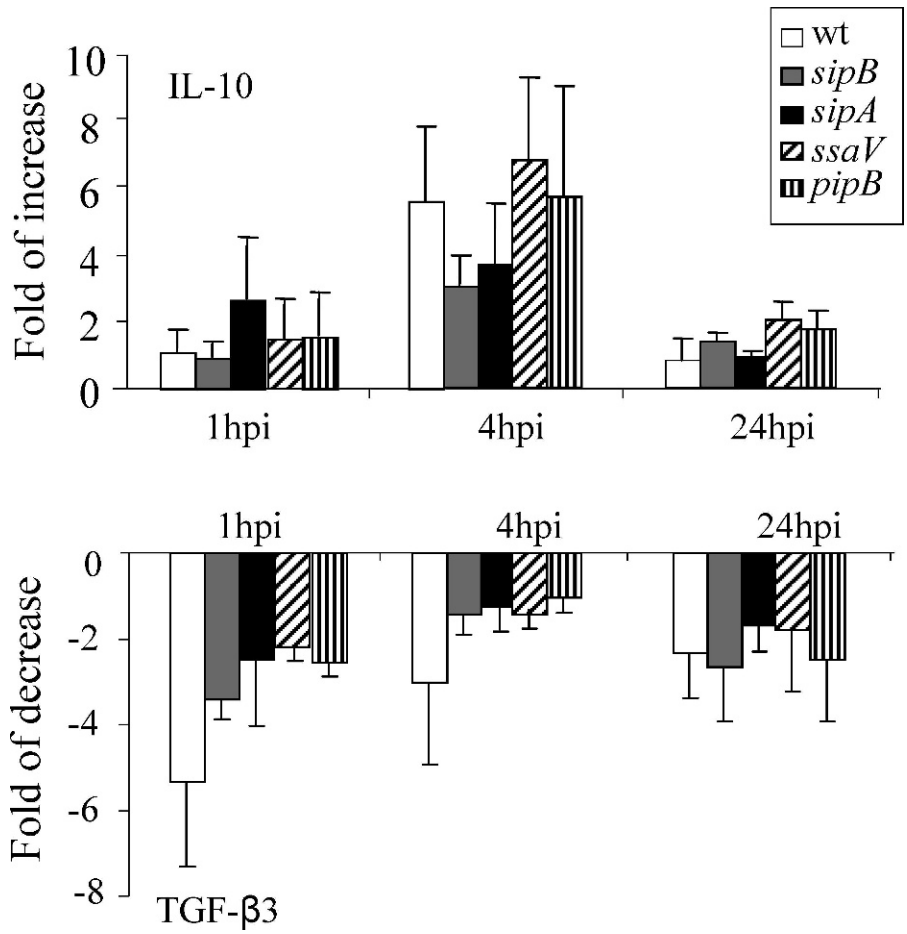

Fig. 3. SE-induced changes in the expression of anti-inflammatory cytokines. The amount of cytokine transcripts in SE-infected COEC relative to that in the control was determined by RT-PCR and presented as fold increase. Duplicate reactions were included in RT-PCR. Data shown (means ± SD) are from three independent experiments.

COEC including inducible nitric oxide synthase (iNOS), CXCLi1 (K60), CXCLi2 (IL-8), CCLi3 (K203), and CCLi4 (Mip1β), although mutations in T3SS-1 and T3SS-2 had different impacts on the expression of these genes. The peak induction of these immune genes in COEC by SE occurred mostly at 4 hpi. At this time point, the amounts of iNOS mRNA transcripts in COEC cultures infected with T3SS mutants (except *ssaV*) were significantly lower than that in COEC infected with the wild-type SE (Fig. 1). A linear correlation ($r = 0.8197$, $P = 0.022$) between the expression levels of iNOS and the numbers of intracellular bacteria in COEC at 4 hpi was detected. With regard to proinflammatory chemokine genes, CXCLi1 (K60) and CXCLi2 (IL-8) genes were more responsive than CCLi3 and CCLi4 to SE, as evidenced by the higher-fold increase in CXC chemokine mRNA (>15-fold) than CC chemokines (<10-fold) at 4 hpi (Fig. 2). The two T3SS-1 mutants, ZM101 (*sipB*) and ZM103 (*sipA*), had significantly reduced ability, compared to the wild-type SE, to stimulate the expression of CXCLi1 at 24 hpi and CXCLi2 at 4 hpi (Fig. 2). In contrast, the two T3SS-2 mutants, ZM102 (*ssaV*) and ZM106 (*pipB*), did not exhibit any defects in the induction of CXCLi1 and CXCLi2 in COEC, compared to ZM100. ZM101 (*sipB*), a strain severely impaired in invasion, also induced lesser amounts of CCLi3 mRNA transcripts than the wild-type SE did. Other T3SS mutants, including ZM102 (*ssaV*), ZM103 (*sipA*), and ZM106 (*pipB*), elicited CCLi3 mRNA expression in COEC at similar levels to that induced by the wild-type SE. None of the T3SS mutants demonstrated any defect in induction of CCLi4 mRNA expression in COEC, compared to the wild-type SE. No linear correlation between the number of intracellular bacteria and the mRNA expression of CXC and CC chemokines in COEC was found among infections ($P > 0.05$). The reduced mRNA expression of CXCLi1 and CXCLi2 in COEC cultures infected with T3SS-1 mutants was not associated with altered COEC viability or death as evidenced by the similar levels of apoptosis and necrosis among infection groups.

SE infections differentially regulated the genes encoding anti-inflammatory cytokines in COEC, with IL-10 being induced and transforming growth factor (TGF)-β3 being suppressed (Fig. 3). However, no significant difference in the ability of individual SE strains to induce IL-10 or suppress TGF-β3 mRNA expression in COEC was detected. Although IL-1β, INF-α, and INF-β mRNA expressions in COEC were detected by RT-PCR, these were not consistently affected by SE infections (data not shown).

SipA-dependent induction of CXC chemokine. Although the intracellular bacterial load was higher in COEC infected with ZM103 (*sipA*) than that in COEC infected with ZM101 (*sipB*), there was no difference in the expression of CXC chemokines between the COEC cultures infected with these mutants. Because one of the main functions of SipB is to facilitate the tranlocation of T3SS-1 effectors into host cells, the reduced ability of the *sipB* mutant to induce CXC chemokine expression was likely due to the failure of this strain to secrete SipA into COEC. To verify the role of SipA in induction of CXC chemokine in COEC, RT-PCR was conducted to assess CXCLi1 and CXCLi2 mRNA expressions in COEC infected with ZM103C, the *sipA* mutant carrying a plasmid

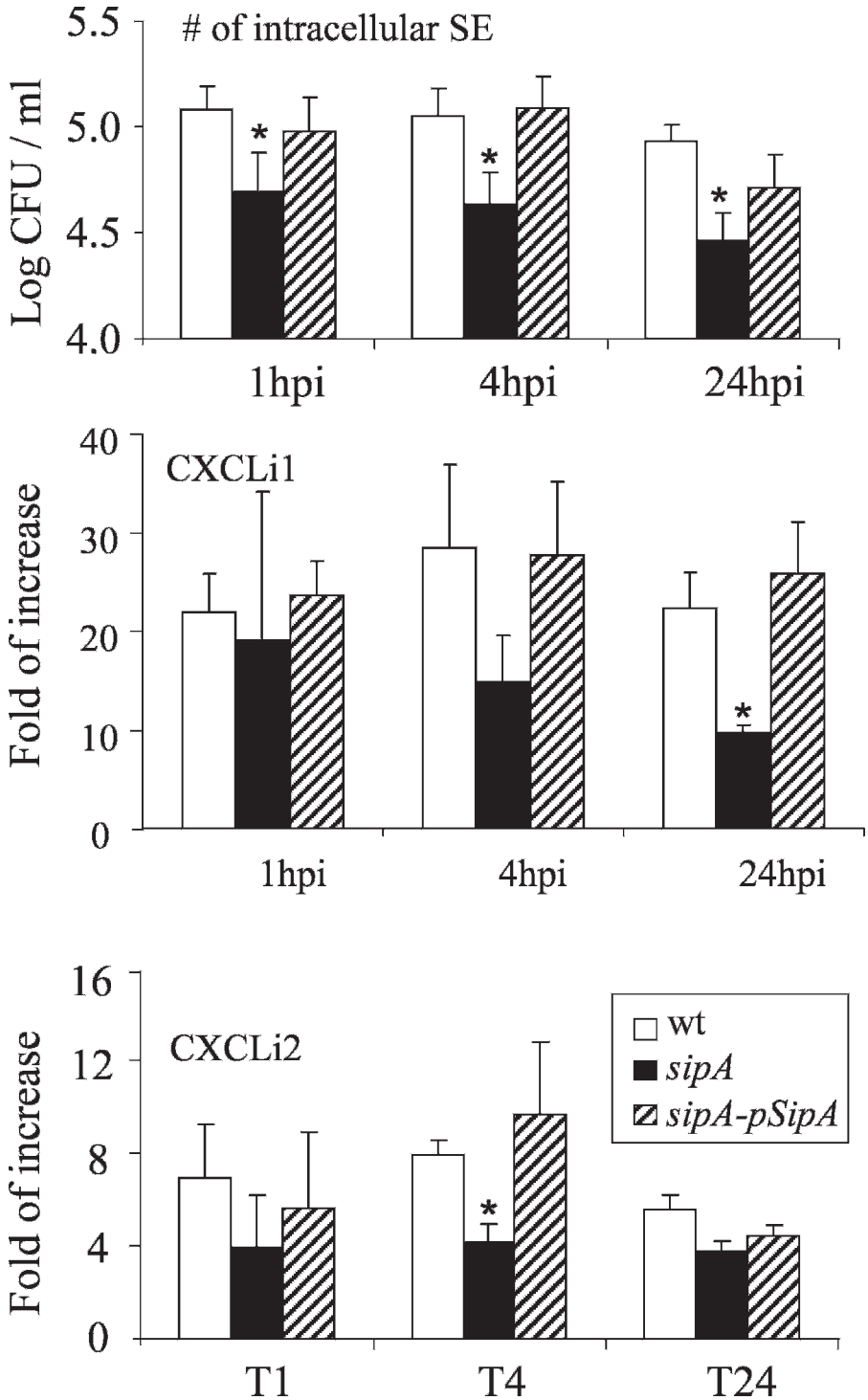

Fig. 4. Genetic complementation of the *sipA* mutation. COEC were infected with wild-type or *sipA*- or *sipA-pSipA*–mutant SE at an MIO of 20:1. The number of intracellular bacteria recovered from COEC was presented as $\log_{10}$ CFU/ml. The amount of CXCLi1 or CXCLi2 transcripts in SE-infected COEC relative to that in the control was determined by RT-PCR and presented as fold increase. Duplicate reactions were included in RT-PCR. Data shown (means ± SD) are from three independent experiments. * indicates the difference between the wild type and the mutant was significant ($P < 0.05$).

expressing the full-length *sipA* gene. The results showed that ZM103-C elicited CXCLi1 and CXCLi2 at levels comparable to that induced by the wild-type SE (Fig. 4). The data indicated that SipA, an effector protein translocated by T3SS-1, was responsible for stimulating the expression of these CXC chemokines during SE infection of COEC.

DISCUSSION

Chemokines and cytokines are important mediators and/or regulators of inflammation and immunity (9). Elevated expression of proinflammatory chemokines and cytokines in chicken intestinal epithelium coincides with bacterial clearance (31). To understand

the complex interplay between bacterial virulence factors and host innate immunity during SE infection of the chicken reproductive tract, we determined the expression profiles of selected chemokines and cytokines in primary COEC exposed to different SE strains. The *sipA* and *pipB* mutants have been chosen because these genes are involved in the entry and survival of SE in COEC and SipA is a potent inducer of CXC chemokine in mammalian hosts (18,27). The *sipB* and *ssaV* mutants were used as control strains because they were deficient in translocating SipA and PipB, respectively (10,13,29). Data from this study showed that COEC are able to launch a proinflammatory response following *in vitro* exposure to SE, indicating an important role of COEC in local innate immunity against SE. Similar degrees of apoptosis and necrosis of COEC among treatment groups confirmed that differential induction of immune genes by T3SS mutants was not associated with any alterations in COEC viability. In fact, we observed that SE induction of iNOS, but not of CC and CXC chemokines, was governed by the number of intracellular bacteria in COEC. This phenomenon suggests that the expression of iNOS was influenced by the amount of bacterial components present, likely lipopolysaccharides and other pathogen-associated molecular patterns (PAMP), in COEC whereas the transcription of chemokines was subjective to the presence of bacterial virulence factors, such as T3SS-1 effectors. However, our data did not rule out the possibility that T3SS effectors were involved in eliciting iNOS expression, but such activity was masked by the potent stimulatory effect of bacterial PAMP.

It has been shown that SipA induces the expression of CXC chemokines in bovine intestinal epithelium and subsequent infiltration of polymorphonuclear leukocytes (PMN) to the site of infection (34). The interaction of SipA with the apical surface of human intestinal epithelial cells can cause PMN transepithelial migration (28). The SipA-dependent induction of CXC chemokines in COEC *per se* resembles what has been observed during *Salmonella* infection of mammalian epithelial cells, suggesting a conserved activity of SipA to interact with host innate immune system. However, the biologic outcomes of SipA-dependent induction of CXC chemokines may be different between mammalian and avian host species. CXC chemokines expressed by bovine or human intestinal epithelium recruit PMN to the site of infection, leading to localized inflammation and tissue damage (17,34). In contrast, avian CXCLi1 (K60) targets monocytes, macrophages, and lymphocytes, instead of heterophils (19,31). A recent *in vivo* study shows that induction of CXCLi1 (K60) and CXCLi2 (IL-8) by SE in chicken intestine correlates to a monocytic, but not heterophilic, type of inflammation (6). Given the fact that *Salmonella* actively resists the killing by macrophages, SipA-dependent induction of CXC chemokines may benefit bacterial survival and spread, rather than clearance. However, the biologic significance of SipA-induction of CXC chemokine during SE colonization of chicken oviduct epithelium should be further studied using *in vivo* infection models and at the protein level due to potential discrepancies between the amounts of mRNA transcribed and protein released. The expression of certain avian cytokines and chemokines is mediated by ERK1/2-dependent activation of AP-1 and NF-κB signaling pathways (16). At the present time, it is not known how SipA differentially targets the transcription of CXC and CC chemokines in mammalian or avian cells. Further investigations aimed at deciphering the mechanism (or mechanisms) involved in SipA induction of CXC chemokines need to be conducted.

In this study, reduced expression of chemokines in COEC was also observed following exposure to the *sipB* mutant, compared to the wild-type SE. The SipB protein of *Salmonella* functions as a translocase as well as an effector (35). Under the current experimental conditions, the function (or functions) of *sipB*, if any, in modulating CXC chemokine expression can not be separated from the pivotal role of this gene in translocation and host cell invasion. Thus, no attempt was made to complement the impaired induction of CXC chemokines by ZM101. The present study did not find any significant roles of T3SS-2 in modulating CC or CXC chemokine expression in COEC.

REFERENCES

1. Barrow, P. A. Experimental infection of chickens with Salmonella enteritidis. Avian Pathol. 20:145–153. 1991.

2. Barrow, P. A., and M. A. Lovell. Experimental infection of egg-laying hens with Salmonella enteritidis phage type 4. Avian Pathol. 20:335–348. 1990.

3. Bohez, L., R. Ducatelle, F. Pasmans, N. Botteldoorn, F. Haesebrouck, and F. Van Immerseel. Salmonella enterica serovar Enteritidis colonization of the chicken caecum requires the HilA regulatory protein. Vet. Microbiol. 116:202–210. 2006.

4. Bohez, L., I. Gantois, R. Ducatelle, F. Pasmans, J. Dewulf, F. Haesebrouck, and F. Van Immerseel. The Salmonella pathogenicity island 2 regulator ssrA promotes reproductive tract but not intestinal colonization in chickens. Vet. Microbiol. 126:216–224. 2008.

5. Chakravortty, D., I. Hansen-Wester, and M. Hensel. Salmonella pathogenicity island 2 mediates protection of intracellular Salmonella from reactive nitrogen intermediates. J. Exp. Med. 195:1155–1166. 2002.

6. Cheeseman, J. H., N. A. Levy, P. Kaiser, H. S. Lillehoj, and S. J. Lamont. Salmonella Enteritidis-induced alteration of inflammatory CXCL chemokine messenger-RNA expression and histologic changes in the ceca of infected chicks. Avian Dis. 52:229–234. 2008.

7. Cox, N. A., J. S. Bailey, J. M. Mauldin, and L. C. Blankenship. Presence and impact of Salmonella contamination in commercial broiler hatcheries. Poult. Sci. 69:1606–1609. 1990.

8. De Buck, J., F. Pasmans, F. Van Immerseel, F. Haesebrouck, and R. Ducatelle. Tubular glands of the isthmus are the predominant colonization site of Salmonella enteritidis in the upper oviduct of laying hens. Poult. Sci. 83:352–358. 2004.

9. Eckmann, L., and M. F. Kagnoff. Cytokines in host defense against Salmonella. Microbes Infect. 3:1191–1200. 2001.

10. Ehrbar, K., S. Mirold, A. Friebel, S. Stender, and W. D. Hardt. Characterization of effector proteins translocated via the SPI1 type III secretion system of Salmonella typhimurium. Review. Int. J. Med. Microbiol. 291:479–485. 2002.

11. Gast, R. K., and P. S. Holt. Persistence of Salmonella enteritidis from one day of age until maturity in experimentally infected layer chickens. Poult. Sci. 77:1759–1762. 1998.

12. Gast, R. K., and P. S. Holt. The relationship between the magnitude of the specific antibody response to experimental Salmonella enteritidis infection in laying hens and their production of contaminated eggs. Avian Dis. 45:425–431. 2001.

13. Hensel, M., J. E. Shea, S. R. Waterman, R. Mundy, T. Nikolaus, G. Banks, A. Vazquez-Torres, C. Gleeson, F. C. Fang, and D. W. Holden. Genes encoding putative effector proteins of the type III secretion system of Salmonella pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. Mol. Microbiol. 30:163–174. 1998.

14. Hoop, R. K., and A. Pospischil. Bacteriological, serological, histological and immunohistochemical findings in laying hens with naturally acquired Salmonella enteritidis phage type 4 infection. Vet. Rec. 133:391–393. 1993.

15. Jones, M. A., S. D. Hulme, P. A. Barrow, and P. Wigley. The Salmonella pathogenicity island 1 and Salmonella pathogenicity island 2 type III secretion systems play a major role in pathogenesis of systemic disease and gastrointestinal tract colonization of Salmonella enterica serovar Typhimurium in the chicken. Avian Pathol. 36:199–203. 2007.

16. Kogut, M. H., K. J. Genovese, H. He, and P. Kaiser. Flagellin and lipopolysaccharide up-regulation of IL-6 and CXCLi2 gene expression in

chicken heterophils is mediated by ERK1/2-dependent activation of AP-1 and NF-kappaB signaling pathways. Innate Immun. 14:213–222. 2008.

17. Lee, C. A., M. Silva, A. M. Siber, A. J. Kelly, E. Galyov, and B. A. McCormick. A secreted Salmonella protein induces a proinflammatory response in epithelial cells, which promotes neutrophil migration. Proc. Natl. Acad. Sci. U. S. A. 97:12283–12288. 2000.

18. Li, S., Z. Zhang, L. Pace, H. Lillehoj, and S. Zhang. Functions exerted by the virulence-associated type three secretion systems during Salmonella enterica serovar Enteritidis invasion into and survival within chicken oviduct epithelial cells and macrophages. Avian Pathol. 38:97–106. 2009.

19. Martins-Green, M. The chicken chemotactic and angiogenic factor (cCAF), a CXC chemokine. Int. J. Biochem. Cell Biol. 33:427–432. 2001.

20. Nakamura, M., N. Nagamine, T. Takahashi, S. Suzuki, M. Kijima, Y. Tamura, and S. Sato. Horizontal transmission of Salmonella enteritidis and effect of stress on shedding in laying hens. Avian Dis. 38:282–288. 1994.

21. Okamura, M., H. S. Lillehoj, R. B. Raybourne, U. S. Babu, and R. A. Heckert. Cell-mediated immune responses to a killed Salmonella enteritidis vaccine: lymphocyte proliferation, T-cell changes and interleukin-6 (IL-6), IL-1, IL-2, and IFN-gamma production. Comp. Immunol. Microbiol. Infect. Dis. 27:255–272. 2004.

22. Okamura, M., H. S. Lillehoj, R. B. Raybourne, U. S. Babu, R. A. Heckert, H. Tani, K. Sasai, E. Baba, and E. P. Lillehoj. Differential responses of macrophages to Salmonella enterica serovars Enteritidis and Typhimurium. Vet. Immunol. Immunopathol. 107:327–335. 2005.

23. Patrick, M. E., P. M. Adcock, T. M. Gomez, S. F. Altekruse, B. H. Holland, R. V. Tauxe, and D. L. Swerdlow. Salmonella enteritidis infections, United States, 1985–1999. Emerg. Infect. Dis. 10:1–7. 2004.

24. Ruiz-Albert, J., R. Mundy, X. J. Yu, C. R. Beuzón, and D. W. Holden. SseA is a chaperone for the SseB and SseD translocon components of the Salmonella pathogenicity-island-2-encoded type III secretion system. Microbiology 149:1103–1111. 2003.

25. Sadeyen, J. R., J. Trotereau, P. Velge, J. Marly, C. Beaumont, P. A. Barrow, N. Bumstead, and A. C. Lalmanach. Salmonella carrier state in chicken: comparison of expression of immune response genes between susceptible and resistant animals. Microbes Infect. 6:1278–1286. 2004.

26. Uchiya, K., E. A. Groisman, and T. Nikai. Involvement of Salmonella pathogenicity island 2 in the up-regulation of interleukin-10 expression in macrophages: role of protein kinase A signal pathway. Infect. Immun. 72:1964–1973. 2004.

27. Vazquez-Torres, A., and F. C. Fang. Salmonella evasion of the NADPH phagocyte oxidase. Microbes Infect. 3:1313–1320. 2001.

28. Wall, D. M., W. J. Nadeau, M. A. Pazos, H. N. Shi, E. E. Galyov, and B. A. McCormick. Identification of the Salmonella enterica serotype typhimurium SipA domain responsible for inducing neutrophil recruitment across the intestinal epithelium. Cell Microbiol. 9:2299–2313. 2007.

29. Waterman, S. R., and W. D. Holden. Functions and effectors of the Salmonella pathogenicity island 2 type III secretion system. Review. Cell Microbiol. 5:501–511. 2003.

30. Wigley, P., S. Hulme, L. Rothwell, N. Bumstead, P. Kaiser, and P. Barrow. Macrophages isolated from chickens genetically resistant or susceptible to systemic salmonellosis show magnitudinal and temporal differential expression of cytokines and chemokines following Salmonella enterica challenge. Infect. Immun. 74:1425–1430. 2006.

31. Withanage, G. S., P. Wigley, P. Kaiser, P. Mastroeni, H. Brooks, C. Powers, R. Beal, P. Barrow, D. Maskell, and I. McConnell. Cytokine and chemokine responses associated with clearance of a primary Salmonella enterica serovar typhimurium infection in the chicken and in protective immunity to rechallenge. Infect. Immun. 73:5173–5182. 2005.

32. Xing, Z., and K. A. Schat. Expression of cytokine genes in Marek's disease virus-infected chickens and chicken embryo fibroblast cultures. Immunology 100:70–76. 2000.

33. Zhang, S., L. G. Adams, J. Nunes, S. Khare, R. M. Tsolis, and A. J. Bäumler. Secreted effector proteins of Salmonella enterica serotype typhimurium elicit host-specific chemokine profiles in animal models of typhoid fever and enterocolitis. Infect. Immun. 71:4795–4803. 2003.

34. Zhang, S., R. L. Santos, R. M. Tsolis, S. Stender, W. D. Hardt, A. J. Bäumler, and L. G. Adams. The Salmonella enterica serotype typhimurium effector proteins SipA, SopA, SopB, SopD, and SopE2 act in concert to induce diarrhea in calves. Infect. Immun. 70:3843–3855. 2002.

35. Zhou, D., and J. E. Galán. Salmonella entry into host cells: the work in concert of type III secreted effector proteins. Review. Microbes Infect. 3:1293–1298. 2001.

36. Ziprin, R. L., and M. H. Kogut. Efficacy of two avian Salmonella-immune lymphokines against liver invasion in chickens by Salmonella serovars with different O-group antigens. Avian Dis. 41:181–186. 1997.

ACKNOWLEDGMENTS

The authors would like to thank Drs. Robert Wills and Hart Bailey for critical review of the manuscript and in-depth discussions over statistical analysis of the experimental data.